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MATHEMATICAL MODELLING OF ATP, K^+ AND Na^+ INTERACTIONS WITH (Na^+ + K^+)-ATPase OCCURRING UNDER EQUILIBRIUM CONDITIONS *

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Summary

The controlling effect of ATP, K^+ and Na^+ on the rate of (Na^+ + K^+)-ATPase inactivation by 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) is used for the mathematical modelling of the interaction of the effectors with the enzyme under equilibrium conditions.

1. Of a series of conceivable interaction models, designed without conceptual restrictions to describe the effector control of inactivation kinetics, only one fits the experimental data described in a preceding paper.

2. The model is characterized by the coexistence of two binding sites for ATP and the coexistence of two separate binding sites for K^+ and Na^+ on the enzyme-ATP complex. On the basis of this model, the effector parameters fitting the experimental data most closely are estimated by means of non-linear least-squares fits.

3. The apparent dissociation constants for ATP of the enzyme-ATP complex or of the enzyme-$(ATP)_2$ complex are computed to lie near 0.0024 mM and 0.34 mM, respectively, irrespective of whether K^+ and Na^+ were absent or K^+ and K^+ plus Na^+, respectively, were present in the experiments.

4. The origin of the high and the low affinity site for binding of ATP to the (Na^+ + K^+)-ATPase molecule is traced back to the coexistence of two catalytic centres which, although primarily equivalent as to the reactivity of their thiol groups with NBD-Cl, are induced into anticooperative communication by ATP binding and thus show an induced geometric asymmetry.

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* Dedicated to the memory of the late Professor Karl Lohmann who died shortly after his 80th birthday.
Abbreviation: NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole.

5. On the basis of the interaction model outlined under item 2 the apparent dissociation constant for K^+ or Na^+ in the ($K^+ + Na^+$)-liganded enzyme-ATP complex are computed to be 1.7 mM and 3.5 mM, respectively.

6. The conclusions concerning the coexistence of two primarily equivalent but anticooperatively interacting catalytic centres and the coexistence of two separate ionophoric centres for Na^+ and K^+ correspond to the appropriate basic postulates of the flip-flop concept of ($Na^+ + K^+$)-ATPase mechanism.

Introduction

The interaction of ATP, Na^+ and K^+ with ($Na^+ + K^+$)-ATPase is not well understood. The accelerating effect of a preincubation of ($Na^+ + K^+$)-ATPase with ATP on the initial rate of transphosphorylation was interpreted by Märdh and Post [1] to be due to a partial rearrangement of enzyme structure. From studies with irreversible inhibitors, Skou and Hilberg [2] reasoned that ATP has not only an effect on the structure of ($Na^+ + K^+$)-ATPase, but also on the affinities of Na^+ and K^+ to the enzyme. They found it tempting to assume that it is this change in structure which leads to the transport of Na^+ and K^+. More recently, Skou [3] concluded from the effects of ATP, Na^+ and K^+ on the rate of ($Na^+ + K^+$)-ATPase inactivation by *N*-ethylmaleimide that ATP increases the apparent affinity for Na^+ relative to K^+ for the 'Na^+ site' seven to eight times. Unfortunately, a quantitative analysis of the effector control of inactivation kinetics could not be performed since *N*-ethylmaleimide produced a multi-exponential inactivation course and incomplete inhibition both in the absence and the presence of the enzyme effectors [2,3].

In the preceding paper [4], we showed that ATP, K^+ and Na^+, when present either individually or in combination, affected the rate of ($Na^+ + K^+$)-ATPase inactivation by the thiol reagent NBD-Cl. Since ATP, due to the absence of Mg^{2+}, was not hydrolyzed, the observed modifications of the inactivation rate reflected the interaction of the mentioned effectors with ($Na^+ + K^+$)-ATPase under equilibrium conditions. Under all circumstances studied, the inactivation rate followed pseudo first-order kinetics amenable to mathematical modelling. In the present study the inactivation-controlling effect of the effectors as observed at various concentrations is used to reveal the interaction features and to quantitate the affinities of the effectors to the enzyme. To this end, we have designed the smallest conceivable series of models to describe the effector control of inactivation kinetics. One of the models investigated is shown suitable to fit the experimental data and to allow estimation the apparent dissociation constants of the various enzyme-effector complexes involved. The estimated constants in effect corroborate or essentially extend the more qualitative evaluations obtained earlier by a simple plotting procedure [4].

Theory and mathematical procedure

The following reasonable assumptions underlie the mathematical analysis of the experimental data. First, the applied total concentration of the effectors is taken as their effective concentration since the effector concentrations used were in great molar excess over the enzyme concentration. Second, a pseudo-monomolecular reaction of NBD-Cl with the enzyme is assumed, as the thiol

reagent was applied in great molar excess over the thiol groups of the enzyme and as the thiol groups essential for $(Na^+ + K^+)$-ATPase activity behaved like a homogeneous population [4]. More specifically, all NBD-Cl-sensitive enzyme species are assumed to follow first-order inactivation kinetics. Third, the interaction rate of the effectors with the enzyme were higher by orders of magnitude than the inactivation rate produced by reaction of NBD-Cl with the enzyme (refer to ref. 4). Hence, the presence of an equilibrium mixture of differently liganded enzyme species is assumed to exist at any time. Fourth, it is supposed that the formed enzyme-S-NBD derivatives causing activity loss were totally inactive and that the enzyme molecules remaining unmodified during exposure to NBD-Cl showed the same properties as prior to exposure. In line with this assumption, a partially inactivated enzyme batch showed the same K_m value for ATP as the unexposed batch. Fifth, it is assumed that the binding of only one potassium ion or one sodium ion to one of the two or three sites on the enzyme molecule (refer to ref. 5) modified maximally the kinetics of enzyme inactivation by NBD-Cl.

The mathematical analysis of the effector-controlled inactivation kinetics starts from the formulation of a general algorithm. The effector-liganded enzyme molecules are written as

$$[E(L_1)_{m_{1r}} \cdots (L_n)_{m_{nr}}]$$

where E enzyme, $L_1 \ldots L_n$ different ligands (effectors) and m_{jr} the number of ligands of type j ($j = 1 \ldots n$) present in the enzyme complex r. The sum of all complexes existing in the equilibrium is given by

$$[E] \sum_r \prod_{j=1}^{n} [L_j]^{m_{jr}} \cdot K_r$$

where K_r is the binding constant of a special ligand valid for the formation of the enzyme complex r. The effector-controlled inactivation rate of $(Na^+ + K^+)$-ATPase resulting from reaction with NBD-Cl is then described by

$$v(L_1 \ldots L_n) = -\frac{d[E_{tot}]}{dt} = \frac{\sum_r k_r \prod_{j=1}^{n} [L_j]^{m_{jr}} \cdot K_r}{1 + \sum_r \prod_{j=1}^{n} [L_j]^{m_{jr}} \cdot K_r} \cdot [E_{tot}] \qquad (1)$$

where E_{tot} is the total concentration of unmodified, active enzyme molecules present at time t, and k_r is the pseudo first-order rate constant for the reaction of NBD-Cl with the enzyme complex r. So, the validity of this algorithm covers first-order inactivation kinetics as experimentally observed [4].

Proceeding from the general algorithm, various expressions for $v(L_1 \ldots L_n)$ were formulated by designing without conceptual restrictions a series of different models eventually appropriate for the description of the effector control of inactivation kinetics. The models VI and X (the latter being an extended form of VI) fitted the experimental data and were thus selected. For the estimate of the effector parameters, nonlinear least-squares fits were performed by means of the Marquardt algorithm as adapted to and computerized for the analysis of

enzyme kinetics by Reich et al. [6]. The applied computer programme ensured discrimination between rival models on the basis of the sums of least squares and permitted the estimate of a set of parameter values with corresponding ranges of statistical variation (refer to ref. 6).

Results and Discussion

As a cogent consequence of the experimental approach, the modelling of the effector control of enzyme inactivation considers only those of the conceivable enzyme-effector complexes which react with NBD-Cl at different rates and thus account for the observed control of the overall inactivation rate by the effectors. Only minimum reaction models including the lowest number of possible complexes are designed to ensure the rigid estimate of parameter values from the data sets reported earlier [4]. In the best fitting models VI and X, the root mean square of the deviation of the computed lines from the experimental points is found to compare favourably with experimental error.

1. Modelling of ATP- and K^+-effected control of enzyme inactivation by NBD-Cl

The starting point of modelling is given by the following observations detailed in the preceding paper [4]. In the absence of ATP, KCl concentrations higher than 10—20 mM KCl, are required to accelerate inactivation. In the presence of low ATP concentrations, however, KCl concentrations as low as 0.3—0.5 mM already suffice to significantly increase the inactivation rate. On the other hand, high ATP concentrations reduce the reinforcing effect of K^+ on rate of enzyme inactivation.

For modelling of the control effect of 0.05—2 mM ATP and 0—3 mM KCl on enzyme reactivity towards NBD-Cl, a series of interaction models was designed from which the following apparently plausible models could be excluded because they were found to be insufficient for fitting the experimental data and for estimating the desired parameter values. The unsuitability of the models I—III

$$E \underset{-K^+}{\overset{+K^+}{\rightleftharpoons}} E \cdot K^+ \underset{-ATP}{\overset{+ATP}{\rightleftharpoons}} ATP \cdot E \qquad \text{I}$$

$$ATP \cdot E \underset{+ATP}{\overset{-ATP}{\rightleftharpoons}} E \underset{-K^+}{\overset{+K^+}{\rightleftharpoons}} E \cdot K^+ \underset{-ATP}{\overset{+ATP}{\rightleftharpoons}} ATP \cdot E \cdot K^+ \qquad \text{II}$$

$$E \cdot K^+ \underset{+K^+}{\overset{-K^+}{\rightleftharpoons}} E \underset{-ATP}{\overset{+ATP}{\rightleftharpoons}} ATP \cdot E \underset{-K^+}{\overset{+K^+}{\rightleftharpoons}} ATP \cdot E \cdot K^+ \qquad \text{III}$$

apparently means that the observed accelerating effect of K^+ on the rate of enzyme inactivation by NBD-Cl is not caused by binding of K^+ to free enzyme as assumed in I or to both free enzyme and enzyme-ATP complex as assumed in II and III. The rejection of models IV and V

$$E \underset{-ATP}{\overset{+ATP}{\rightleftharpoons}} ATP \cdot E \underset{-K^+}{\overset{+K^+}{\rightleftharpoons}} ATP \cdot E \cdot K^+ \qquad \text{IV}$$

$$E \underset{-ATP}{\overset{+ATP}{\rightleftharpoons}} ATP \cdot E \underset{-K^+}{\overset{+K^+}{\rightleftharpoons}} ATP \cdot E \cdot K^+ \underset{-ATP}{\overset{+ATP}{\rightleftharpoons}} (ATP)_2 \cdot E \cdot K^+ \qquad V$$

and the fitness of model VI (see below) show that an enzyme-$(ATP)_2$ complex lacking in IV must be included, but that binding of K^+ to this complex as assumed in V is not involved in causing the K^+ effect. The fitness of the resulting model VI

$$\begin{array}{ccccc} & & (ATP)_2 \cdot E & & \\ & & \Big\uparrow \Big\downarrow & & \\ E & \underset{-ATP}{\overset{+ATP}{\rightleftharpoons}} & ATP \cdot E & \underset{-K}{\overset{+K^+}{\rightleftharpoons}} & ATP \cdot E \cdot K^+ \\ \Big\downarrow k_1 & K_S & \Big\downarrow k_2 & K_{K^+} & \Big\downarrow k_3 \end{array} \qquad VI$$

apparently means that the accelerating effect of K^+ on the reaction of NBD-Cl with the enzyme is due to shifting of the equilibrium between the enzyme-$(ATP)_2$ complex and the K^+-liganded enzyme-ATP complex to the side of the latter. In this model, consequently, free enzyme, enzyme-ATP complex and K^+ liganded enzyme-ATP complex are assumed to react with NBD-Cl at different rates characterized by the pseudo first-order rate constants k_1, k_2 and k_3, while the enzyme-$(ATP)_2$ complex remains unreactive, since the essential thiol groups in the catalytic centres are protected from NBD-Cl attack (see [4]).

For the estimate of the desired parameter values K_S, K_{S_2} and K_{K^+}, the following equation based on model VI was used

$$\frac{v(ATP, K^+)}{v_0} = \frac{1 + \frac{k_2}{k_1}\frac{ATP}{K_S} + \frac{k_3}{k_1}\frac{[ATP][K^+]}{K_S K^+}}{1 + \frac{[ATP]}{K_S} + \frac{[ATP]^2}{K_S K_{S_2}} + \frac{[ATP][K^+]}{K_S K_{K^+}}} \qquad (2)$$

It describes the ratio of the rates of enzyme inactivation in presence of ATP and K^+ ($v(ATP, K^+)$) and in absence of these effectors (v_0) as a function of the effector concentrations and the various kinetic parameters. The combination of parameter values listed in Table I yielded the best fit of computed and experi-

TABLE I

COMBINATION OF PARAMETER VALUES YIELDING BEST FIT OF COMPUTED AND EXPERIMENTAL DATA FOR THE EQUILIBRIA BETWEEN ENZYME, ATP AND K^+

The parameter values were computed on the basis of model VI by means of eq. 2. The table shows the dissociation constants for ATP in the enzyme-ATP complex (K_S) or in the enzyme-$(ATP)_2$ complex (K_{S_2}), and for K^+ in the K^+-liganded enzyme-ATP complex (K_{K^+}) as well as the ratios of the pseudo first-order rate constants of NBD-Cl reaction with different enzyme states (k_2/k_1 and k_3/k_1).

Parameter	Parameter value	Statistical variation
K_S	0.0027 mM	±30%
K_{S_2}	0.42 mM	±23%
K_{K^+}	2.2 mM	±40%
k_2/k_1	0.45	±8%
k_3/k_1	0.63	±22%

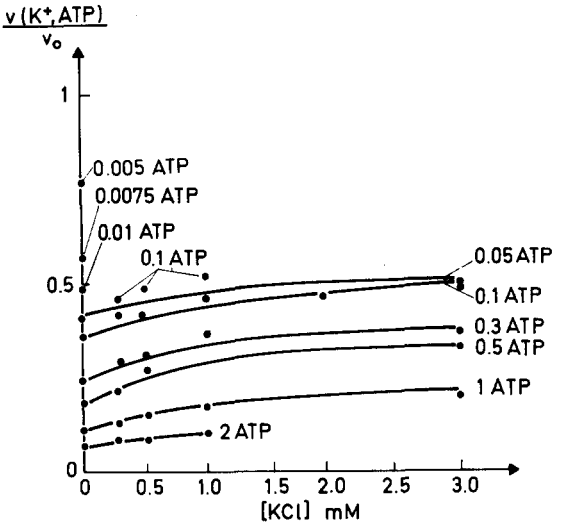


Fig. 1. Influence of variant concentrations of KCl and ATP (given in mM) on the ratio of the rates of enzyme inactivation by NBD-Cl in presence of KCl and ATP ($v(K^+, ATP)$) and in absence of effectors (v_0). The symbols or curves refer to the experimental and computed data, respectively. The calculations were done on the basis of model VI by means of equation 2. At 0.005, 0.0075 and 0.01 mM ATP, the experimental and computed values coincided and are thus not differentiated on the figure.

mental data which are compared in Fig. 1. The deviations from the fit found with 0.1 mM ATP are probably due to experimental error since no trends are seen in the residual data pairs. The satisfactory fit of computed and observed data (Fig. 1) and the reasonable degree of statistical variation of estimated parameter values (Table I) suggest both the competence of the applied reaction model VI and the relevance of the estimates of K_S, K_{S_2} and K_{K^+} shown in Table I.

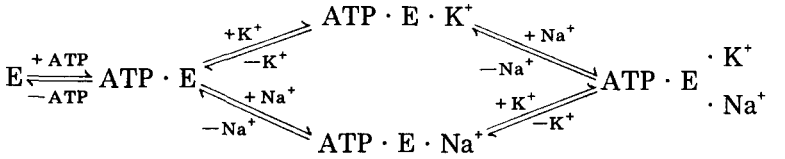
2. Modelling of ATP-, K^+- and Na^+-effected control of enzyme inactivation by NBD-Cl

The starting point of modelling is given by the following observations which for the most part were fully described in the preceding paper [4]. In presence of 0.1 or 2.0 mM ATP, the addition of NaCl (1—30 mM) does not affect the rate of enzyme inactivation by NBD-Cl (not demonstrated). However, in the presence of both ATP (0.5 mM) and KCl (0.3—6 mM), the addition of NaCl in increasing concentrations progressively reverses the accelerating effect of K^+ on the rate of enzyme inactivation so that with 20—30 mM NaCl the resulting rate is even slower than in the presence of ATP alone. These observations favour the interpretation that the Na^+-produced reversal of the K^+ effect on enzyme inactivation is not due to competitive displacement of K^+ by Na^+ from a single binding site on the enzyme, but results from the simultaneous binding of both Na^+ and K^+ to two separate sites coexisting on the enzyme-ATP complex.

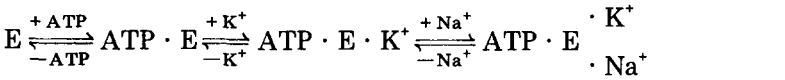
In line with this interpretation, the reaction model VII

$$\begin{array}{ccccc} & & (ATP)_2 \cdot E & & \\ & & {\scriptstyle -ATP}\ \big\downarrow\big\uparrow\ {\scriptstyle +ATP} & & \\ E & \underset{-ATP}{\overset{+ATP}{\rightleftharpoons}} & ATP \cdot E & \underset{-K^+}{\overset{+K^+}{\rightleftharpoons}} & ATP \cdot E \cdot K^+ \\ & & {\scriptstyle +Na^+}\ \big\downarrow\big\uparrow\ {\scriptstyle -Na^+} & & \\ & & ATP \cdot E \cdot Na^+ & & \end{array} \qquad \text{VII}$$

assuming a competitive displacement of K^+ by Na^+ from a common binding site, was found to be inadequate for fitting the experimental data and for estimating the desired parameter values. The unsuitability of models VIII and IX

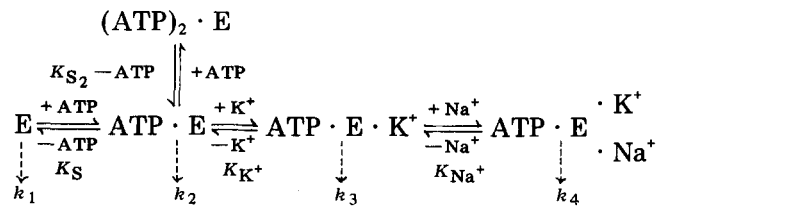


VIII



IX

and the fitness of model X which extends model VI



X

apparently mean that the enzyme-$(ATP)_2$ complex has to be included and that the Na^+-produced reversal of the accelerating K^+ effect on the rate of enzyme inactivation must result from the formation of the Na^+- plus K^+-liganded enzyme-ATP complex which should show a much reduced reactivity toward NBD-Cl (characterized by the pseudo first-order rate constant, k_4) compared to the reactivity of the other enzyme states (characterized by k_1, k_2 and k_3) except the totally unreactive enzyme-$(ATP)_2$ complex.

For the estimate of the desired parameter values K_S, K_{S_2}, K_{K^+} and K_{Na^+}, the following equation based on model X was used:

$$\frac{v(\mathrm{ATP}, K^+, Na^+)}{v_0} = \frac{1 + \frac{[\mathrm{ATP}]}{K_S}\left(\frac{k_2}{k_1} + \frac{k_3}{k_1}\frac{[K^+]}{K_{K^+}} + \frac{k_4}{k_1}\frac{[Na^+][K^+]}{K_{Na^+}K_{K^+}}\right)}{1 + \frac{[\mathrm{ATP}]}{K_S} + \frac{[\mathrm{ATP}]^2}{K_S K_{S_2}} + \frac{[\mathrm{ATP}]}{K_S}\left(\frac{[K^+]}{K_{K^+}} + \frac{[Na^+][K^+]}{K_{Na^+}K_{K^+}}\right)}. \qquad (3)$$

Since model X is but an extension of model VI and since the rates of enzyme inactivation by NBD-Cl as function of [ATP] and $[K^+]$ ($v(\mathrm{ATP}, K^+)$) as well as function of [ATP], $[K^+]$ and $[Na^+]$ ($v(\mathrm{ATP}, K^+, Na^+)$) were normalized by relating them to the rate of inactivation in absence of effectors (v_0) (see Eqns. 2 and 3), the data set obtained with ATP and K^+ was included in the estimate of parameter values by means of Eqn. 3. Hence the fitting procedure on the basis of model X involved the controlling effect of 0—2 mM ATP, 0—6 mM KCl and 0—30 mM NaCl on the rate of enzyme inactivation. The combination of parameter values listed in Table II yields the best fit of computed and experimental data which are partially compared in Fig. 2. The rather high statistical variation of k_4/k_1 is simply explained by the very low value of k_4. The good fit

TABLE II

COMBINATION OF PARAMETER VALUES YIELDING BEST FIT OF COMPUTED AND EXPERIMENTAL DATA FOR THE EQUILIBRIA BETWEEN ENZYME, ATP, K^+ AND Na^+

The parameter values were computed on the basis of model X by means of eq. 3. The table shows the dissociation constants for ATP in the enzyme-ATP complex (K_S) or in the enzyme-$(ATP)_2$ complex (K_{S_2}), and for K^+(K_{K^+}) or for Na^+(K_{Na^+}) in the K^+-plus Na^+-liganded enzyme-ATP complex as well as the ratios of the pseudo first-order rate constants of NBD-Cl reaction with different enzyme states (k_2/k_1, k_3/k_1 and k_4/k_1).

Parameter	Parameter value	Statistical variation (%)
K_S	0.0024 mM	±36
K_{S_2}	0.34 mM	±20
K_{K^+}	1.7 mM	±48
K_{Na^+}	3.5 mM	±50
k_2/k_1	0.50	±9
k_3/k_1	0.55	±11
k_4/k_1	0.06	±90

of computed and observed data (Fig. 2) and the reasonable degree of statistical variation of estimated parameter values (Table II) suggest both the competence of model X and the relevance of the estimates of K_S, K_{S_2}, K_{K^+} and K_{Na^+} shown in Table II.

3. Mechanistic significance of the results derived from model X

The evaluation has to take into account the fact, not considered in our con-

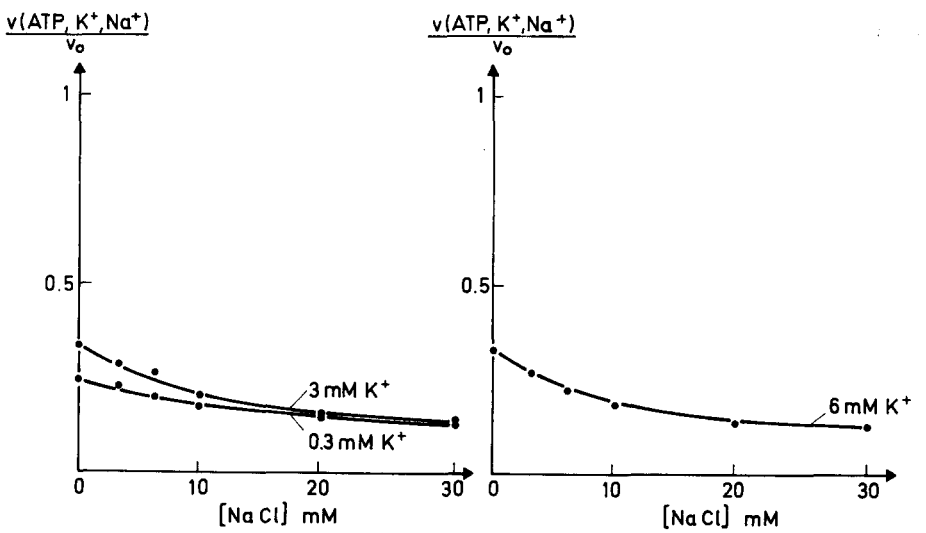


Fig. 2. Influence of variant concentrations of NaCl and KCl in presence of 0.5 mM ATP on the ratio of the rates of enzyme inactivation by NBD-Cl in presence of NaCl, KCl plus ATP (v(ATP, K^+, Na^+)) and in absence of effectors (v_0). The symbols or curves refer to the experimental and computed data, respectively. The calculations were done on the basis of model X by means of Eqn. 3. The influence on the ratio of varying concentrations of ATP (0—2 mM) and of KCl (0—6 mM) in absence of NaCl shown in Fig. 1 is not included as this requires a three-dimensional representation.

ceptually unrestricted model design, that ($Na^+ + K^+$)-ATPase contains two polypeptide chains of molecular weight near 100 000 (refer to ref. 7) which span the membrane and carry both the catalytic and ionophoric centres (Repke, K.R.H., unpublished). In line with this dimeric characteristic, the following analysis of our results suggests the coexistence of two primarily equivalent, but anticooperatively interacting, catalytic centres and of two separate ionophoric centres able to bind simultaneously K^+ and Na^+.

In principle, our finding that ($Na^+ + K^+$)-ATPase shows both high and low affinity to ATP (refer to Table II) could be explained by two alternative models. The first model of preexistent asymmetry assumes primary non-equivalence of two ATP binding sites which, due to non-identity of either primary structure or of conformation and environment, causes highly different affinities for ATP. Although the two chains could not be separated and the NH_2-terminal amino acid sequence showed no heterogeneity [7], differences in the composition of the amino acids forming the two sites are clearly not excluded. However, the pseudo-first-order inactivation rate of enzyme by NBD-Cl [4] indicates complete equivalence of the reacting essential thiol groups known to be located in or near the catalytic centre (see ref. 4). The deduced equivalence is not called into question by the observation that *N*-ethylmaleimide produces a multi-exponential course of enzyme inactivation [2,3] since it is well-established that different thiol reagents can show different reaction patterns. For instance, in glyceraldehyde-3-phosphate dehydrogenase composed of four identical subunits, some thiol reagents react independently with the essential SH groups in the catalytic centres of all subunits, whereas others produce anticooperative reactivity in which modification of the SH groups in two subunits drastically reduces the reactivity of the SH groups in the remaining two subunits [8].

The available evidence thus suggests the aptness of the second model of induced asymmetry in which it is assumed that ($Na^+ + K^+$)-ATPase contains two primarily equivalent, active centres which are brought to anticooperative communication through substrate binding. More specifically, upon high-affinity binding of ATP to one of the two centres, a conformation changes of the proper catalytic chain is produced and transmitted across subunit contacts to the other catalytic chain so that low-affinity binding of ATP to the other centre results. This type of negative cooperativity (see ref. 9) has been already successfully applied to a number of other enzymes [10].

Anticooperativity with respect to one ligand need not imply anticooperativity with respect to all ligands (see ref. 8). So, the analysis of our results with the thiol reagent NBD-Cl carried out and presented by Glynn in the Appendix shows that ATP, when occupying one catalytic centre, does not change the NBD-Cl reactivity of the thiol groups in the other catalytic centre. Apparently, the anticooperativity expressed by low-affinity binding of ATP is not due to weakened hydrogen donation by the thiol group, for the formation of a hydrogen bridge bonding assumed to be involved in ATP binding [11,12], but is caused by a changed geometry of the catalytic centre rendering more difficult ATP binding to it. Our conclusion that the two catalytic centres of ($Na^+ + K^+$)-ATPase are equivalent is at variance with the hypothesis of Jørgensen [13] that the two chains are not identical, but that one chain

contains the ATP binding site and the other has a site for hydrolysis of acyl phosphates. However, the biphasic pattern of tryptic digestion of $(Na^+ + K^+)$-ATPase in presence of 150 mM NaCl underlying the hypothesis could simply have been caused by an asymmetric, conformational change of the enzyme produced by Na^+ binding to only one of the two catalytic chains.

The fact that Na^+ binding to the K^+-liganded enzyme-ATP complex reduces the reactivity of the thiol group in the other catalytic centre not occupied by ATP to almost zero (as indicated by the value of k_4/k_1 given in Table II) requires comment. Since Na^+ binding is required for transphosphorylation from ATP to one catalytic chain, it is probable that Na^+ binds to that chain. If so, Na^+ binding appears to produce there a conformation change which through focussed intersubunit transmission renders the thiol groups in the catalytic centre of the other catalytic chain almost unreactive toward NBD-Cl. This induced interaction between the two subunits allows us to rule out a pre-existent asymmetry model in which absolutely no induced change to the other subunit occurs. Such communications between the two chains are presumably involved in the control of their concerted interplay in the transport work of the enzyme. Similar functions of intersubunit communication were already experimentally verified [8,14].

The method applied to study ligation of $(Na^+ + K^+)$-ATPase with effectors can trace effector-binding only if the formed enzyme-effector complex exhibits a changed reactivity to NBD-Cl. So, binding of Na^+ to enzyme-ATP complex, and to enzyme-$(ATP)_2$ complex, although very probably occurring, could not be detected. Most important, the scrutiny of the alternative models VII—X by fitting of all experimental data showed that ionophoric centres for both Na^+ and K^+ coexist at least in the enzyme-ATP complex. This result subtantiates the assumption derived from studies on transport kinetics that the occurrence of coupled Na^+/K^+-transport requires the simultaneous binding of Na^+ and K^+ to $(Na^+ + K^+)$-ATPase (refer to ref. 15).

In conclusion, model X, initially designed only to describe the experimental data, has a definite mechanistic meaning. Its essential features, namely the coexistence of two primarily equivalent, anticooperatively interacting catalytic centres and the coexistence of separate ionophoric centres for Na^+ and K^+, correspond to the appropriate basic postulates of the flip-flop concept of $(Na^+ + K^+)$-ATPase mechanism [16—18].

4. Physiological significance of estimated dissociation constants

The dissociation constants of the enzyme-ATP complex and the enzyme-

TABLE III

COMPARISON OF THE DISSOCIATION CONSTANTS FOR ATP IN THE ENZYME-ATP COMPLEX (K_S) OR IN THE ENZYME-$(ATP)_2$ COMPLEX (K_{S_2}) FOUND IN THE ABSENCE OR PRESENCE OF CATIONS

Conditions	K_S(μM)	K_{S_2}(μM)	Source of data
Absence of K^+ and Na^+	5 ± 2	240 ± 30	Table I in ref. 4
Presence of K^+	2.7 ± 0.8	420 ± 97	Table I in this paper
Presence of K^+ and Na^+	2.4 ± 0.9	340 ± 68	Table II in this paper

$(ATP)_2$ complex were found not significantly different when the present experiments were done in absence of Na^+ and K^+, in presence of K^+ or in presence of both K^+ and Na^+ (Table III). This shows that the constants reflect an intrinsic design of the enzyme protein expressed by the anticooperative interaction between the catalytic centres of the two catalytic chains. More specifically, the (moderate) influence of variant KCl concentrations on the K_m-(ATP) value of $(Na^+ + K^+)$-ATPase [19] thus cannot be due to a change of the dissociation constant, but must be caused by the K^+ effect on the steady-state concentrations of enzyme intermediates which easily or hardly bind ATP. The low and the high dissociation constant estimated for ATP (Table II) lies in the range of the low and the high K_m(ATP) value of $(Na^+ + K^+)$-ATPase found when the ATP concentration is varied over a wide range. As detailed in the preceding paper [4], the two K_m(ATP) values reflect the absence or presence of coupled interplay between the two catalytic centres concurring with (Na^+)-ATPase and $(Na^+ + K^+)$-ATPase activity, respectively. This behaviour is simulated by the equilibrium model X with the major difference that in the coupled turnover system the anticooperative interaction refers to the substrate ATP and the product ADP which is bound to the catalytic centre almost as tightly as ATP (refer to Table I in ref. 4). The anticooperative coupling between the highly exergonic step of ATP binding and the highly endergonic step of ADP removal contributes to account for the facts that the enzyme system, when effecting coupled Na^+/K^+-transport, works with high speed and high energetic efficiency not far from thermodynamic equilibrium (see refs. 16—18). The anticooperative coupling of two catalytic centres accelerates maximal velocity of $(Na^+ + K^+)$-ATPase 10—20 times (refer to Table II in ref. 4). So, in $(Na^+ + K^+)$-ATPase at least this one of the four advantages which anticooperativity was supposed to provide an enzyme (see ref. 10) is realized.

In order to determine the affinities of ATP, K^+ or Na^+ to $(Na^+ + K^+)$-ATPase, we used enzyme preparations treated to remove any ATP sticking to the high-affinity centre (see refs. 4 and 20). With such nucleotide-depleted preparations, the KCl or NaCl concentrations required to produce half-maximal acceleration or deceleration of the rate of enzyme inactivation by NBD-Cl approach approx. 100 mM [4], whereas in presence of ATP the dissociation constants for K^+ or Na^+ are as low as 1.7 mM and 3.5 mM, respectively (Table II). These findings do not seem to depend on our analytical approach because Ostroy et al. [22] obtained similar results, although using changes in the spin relaxation time of ^{23}Na as measure of Na^+ binding to the enzyme. With a highly purified preparation, Na^+ binding was almost absent, but was easily traceable after addition of ATP. However, with a partially purified preparation, Na^+ binding did not require ATP addition. Taken together, the observations suggest the conclusion that bound ATP (or ADP) was present in all enzyme preparations which showed high-affinity binding of Na^+ or K^+ without added ATP and no dramatic change in cation affinity upon ATP addition (refer to Table IV, Nos. 2—4). If so, ATP binding to $(Na^+ + K^+)$-ATPase produces a structural reorganization of the functional enzyme dimer which leads not only to the anticooperative reciprocation of subunits, but also to the accessibility of high-affinity sites for binding of Na^+ and K^+.

Table IV also shows that among the dissociation constants for Na^+ or K^+

TABLE IV

COMPARISON OF DISSOCIATION CONSTANTS FOR Na^+ OR K^+(K_D VALUES) AND NaCl OR KCl CONCENTRATIONS EFFECTING HALF-MAXIMAL ($Na^+ + K^+$)-ATPase ACTIVITY ($K_{0.5}$ VALUES) FOUND UNDER DIFFERENT CONDITIONS WITH VARIOUS ANALYTICAL APPROACHES

No.	Conditions	Analytical approach and remarks	K_D or $K_{0.5}$ for Na^+ (mM)	K_D or $K_{0.5}$ for K^+ (mM)	Reference
1	0—30 mM NaCl, 0—6 mM KCl, 0—2 mM ATP, pH 7.4, 37°C	Decelerating effect of Na^+-ligation or accelerating effect of K^+-ligation on rate of inactivation by NBD-Cl	3.5	1.7	This paper
2	1—5 mM NaCl (plus 1 mM ATP, 0.4 mM EDTA), pH 7.8, 37°C	Decelerating effect of Na^+-ligation on rate of inactivation by dicyclohexylcarbodiimide	2.3 (2.1)	—	21
3	1—10 mM KCl, 3 mM $MgCl_2$, pH 7.8, 37°C	Accelerating effect of K^+-ligation on rate of inactivation by $BeCl_2$	—	1.4	21
4	20—100 mM NaCl (plus 5 mM ATP), pH 7, 9°C	Changes in the spin lattice relaxation time of ^{23}Na as measure of Na^+ binding	4.0 (7.2)	—	22
5	Constant amount of carrier-free ^{22}Na, 0.1—10 mM NaCl, 1.5 mM $MgCl_2$, pH 7.4, 0°C	Radioactivity due to ^{22}Na binding corrected for amount of radioactivity in water space. Blocked by ouabain. Reduced by KCl. Not affected by ATP	0.23	—	23
6	0.02—20 mM ^{42} KCl, 5 mM $MgCl_2$, pH 7.2, 0°C	Radioactivity due to ouabain-sensitive ^{42}K binding. Halved by 0.2 mM ATP. Eliminated by 100 mM NaCl.	—	0.02, 0.1	24
		Radioactivity due to ouabain-insensitive ^{42}K binding	—	9	
7	Variable [NaCl], 1—10 mM KCl, 3 mM $MgCl_2$, 3 mM ATP, pH 7.8, 37°C	NaCl concentration producing half-maximal ($Na^+ + K^+$)-ATPase activity at given KCl concentration	1.8—5.1	—	25
8	Variable [KCl], 4—90 mM NaCl, 3 mM $MgCl_2$, 3 mM ATP, pH 7.8, 37°C	KCl concentration producing half-maximal ($Na^+ + K^+$)-ATPase activity at given NaCl concentration	—	0.15—0.8	25
9	Variable [NaCl], 0.1—100 mM KCl, 5 mM $MgCl_2$, 2 mM ATP, pH 7.5, 25°C	NaCl concentration producing half-maximal ($Na^+ + K^+$)-ATPase activity at given KCl concentration	3—18.5 6.3 *	—	26
10	Variable [KCl], 5—100 mM NaCl, 5 mM $MgCl_2$, 2 mM ATP, pH 7.5, 25°C	KCl concentration producing half-maximal ($Na^+ + K^+$)-ATPase activity at given NaCl concentration	—	0.1—0.8 0.8 *	26

* $K_{0.5}$ value for optimal conditions.

(Nos. 1—6), two K_D values (Nos. 5, 6) lie more than one order of magnitude below the NaCl or KCl concentrations effecting under optimal conditions half-maximal ($Na^+ + K^+$)-ATPase activity ($K_{0.5}$ values given under Nos. 9 and 10). This discrepancy calls in question that the constants concerned characterize the filling of binding sites which are involved in the transport work of the enzyme. The extraordinarily high affinity sites for Na^+ or K^+ binding found in the presence of $MgCl_2$ (Nos. 5, 6) become eliminated by ouabain binding to the enzyme. The free enthalpy conservation condition leads directly to reciprocity between binding of ouabain and Na^+ or K^+, in the sense that, energetically, the binding of the cation produces upon the binding of ouabain the same effect that the binding of ouabain produces upon the binding of the cation (see ref. 27). Hence, the filling of the concerned binding sites by Na^+ and K^+ could be involved in the modulation of the formation of enzyme-ouabain complex.

The NaCl and KCl concentrations characterizing the dissociation constants of the Na^+- and K^+-liganded enzyme-ATP complex (No. 1 in Table IV) lie near to the NaCl and KCl concentrations effecting half-maximal ($Na^+ + K^+$)-ATPase activity under optimal conditions (Nos. 9, 10). This numerical coincidence favours the conclusion that Na^+ and K^+ binding to the coexisting ionophoric centres of ($Na^+ + K^+$)-ATPase requires the formation of the enzyme-ATP complex which produces a structural reorganization of the enzyme protein and is thus the rate-limiting step under optimal turnover conditions (Repke, K.R.H., unpublished).

Our observation that the K_D value for K^+ remains unchanged by binding of Na^+ to the enzyme-ATP complex (see Tables I and II) means that there is no connexion between the coexisting separate ionophoric centres for K^+ and Na^+. This behaviour of ($Na^+ + K^+$)-ATPase in the equilibrium system corresponds to the response of the Na^+/K^+-transport system in erythrocytes since the external KCl concentration required for half-maximal activation of K^+-transport becomes but very slightly increased as the internal NaCl concentration is raised [28]. In the light of the independence of the ionophoric centres for K^+ and Na^+ found in the equilibrium system, the small increase of the $K_{0.5}$ value for K^+ observed in the transport system does not seem to reflect a connexion between the cation binding sites of the pump enzyme as originally suggested [28], but appears to be due to an effect on transport kinetics.

Appendix — Analysis of the kinetics of ($Na^+ + K^+$)-ATPase inactivation by NBD-Cl in the presence of ATP as a test for the equivalence or non-equivalence of the two binding sites for ATP.

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From an evaluation of the effects of different ATP concentrations on the kinetics of inactivation by NBD-Cl, Grosse et al. [4] concluded that ($Na^+ + K^+$)-ATPase possesses two ATP binding sites differing in their affinities by about two orders of magnitude. They attributed this difference in affinity to anticooperative interaction, but the question of whether the difference resulted from the binding of ATP to two originally equivalent but anticooperative sites or to two primarily nonequivalent sites was not settled. The following analysis

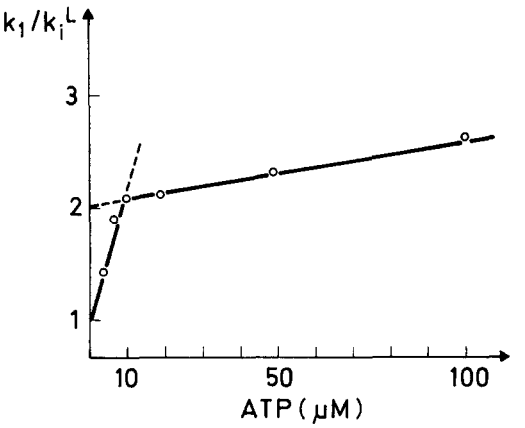

Fig. A-1. The ratio of the rate constants for enzyme inactivation by NBD-Cl in the absence of ATP (k_1) and in the presence of ATP (k_i^L) plotted against the ATP concentration. (From ref. ref. 4).

of their data (reproduced in Fig. A-1) suggests that the two sites are originally equivalent.

If $[E_{tot}]$ is the concentration of uninhibited enzyme in all forms, [S] is the concentration of ATP, k_1 is the rate constant for inhibition by NBD-Cl of free enzyme, k_2 is the rate constant for inhibition of enzyme (ES) binding one molecule of ATP, and we assume that enzyme (ES_2) binding two molecules of ATP is fully protected, then the observed rate constant (k_i^L) for inactivation at any particular concentration of ATP is given by:

$$k_i^L = \{k_1([E_{tot}] - [ES] - [ES_2]) + k_2[ES]\}/[E_{tot}] . \quad (1)$$

Hence

$$k_1/k_i^L = [E_{tot}]/([E_{tot}] - [ES] - [ES_2] + \alpha[ES]) , \quad (2)$$

where $\alpha = k_2/k_1$.

If K_s is the dissociation constant for the reaction $ES \rightleftharpoons E + S$, and K_{s_2} is the dissociation constant for the reaction $ES_2 \rightleftharpoons ES + S$, we can eliminate [ES] and $[ES_2]$ to obtain:

$$k_1/k_i^L = ([S] + [S]^2/K_{s_2} + K_s)/(K_s + \alpha[S]) . \quad (3)$$

When $[S] << K_{s_2}$, and $\alpha[S] << K_s$, which will be true when the ATP concentration is sufficiently low, Eqn. 3 simplifies to:

$$k_1/k_i^L = 1 + [S]/K_s . \quad (4)$$

A graph of k_1/k_i^L against [S] should therefore give a straight line (of slope $1/K_s$) cutting the y-axis at 1. Examination of Fig. 1 shows that the observed behaviour at very low concentrations of ATP is compatible with this prediction.

When $[S] >> K_s$ and $\alpha[S] >> K_s$, which will be true when the ATP concentration is sufficiently high, Eqn; 3 simplifies to:

$$k_1/k_i^L = 1/\alpha + [S]/\alpha K_{s_2} . \quad (5)$$

A graph of k_1/k_i^L against [S] should therefore give a straight line (of slope $1/\alpha K_{s_2}$) cutting the y-axis at $1/\alpha$. Examination of Fig. 1 shows that, at ATP concentrations greater than about 10 μM, the graph is indeed linear, and the intercept is very close to 2. Hence $\alpha = 0.5$; in other words, occupation of one ATP binding site appears to reduce the rate of inactivation by NBD-Cl to precisely one half. This is easily explained if the two ATP binding sites are on originally identical subunits, but would not be expected, except by chance, if the two ATP binding sites were primarily non-equivalent.

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